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Mutation of *toxB* and a Truncated Version of the *efa-1* Gene in *Escherichia coli* O157:H7 Influences the Expression and Secretion of Locus of Enterocyte Effacement-Encoded Proteins but not Intestinal Colonization in Calves or Sheep

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Enterohemorrhagic *Escherichia coli* (EHEC) strains comprise a broad group of bacteria, some of which cause attaching and effacing (AE) lesions and enteritis in humans and animals. Non-O157:H7 EHEC strains contain the gene *efa-1* (referred to in previous publications as *efa1*), which influences adherence to cultured epithelial cells. An almost identical gene in enteropathogenic *E. coli* (*lifA*) mediates the inhibition of lymphocyte proliferation and proinflammatory cytokine synthesis. We have shown previously that significantly lower numbers of EHEC O5 and O111 *efa-1* mutants are shed in feces following experimental infection in calves and that these mutants exhibit reduced adherence to intestinal epithelia compared with isogenic wild-type strains. *E. coli* O157:H7 strains lack *efa-1* but encode a homolog on the pO157 plasmid (*toxB*/*l7095*) and contain a truncated version of the *efa-1* gene (*efa-1′*/*z4332* in O island 122 of the EDL933 chromosome). Here we report that *E. coli* O157:H7 *toxB* and *efa-1′* single and double mutants exhibit reduced adherence to cultured epithelial cells and show reduced expression and secretion of proteins encoded by the locus of enterocyte effacement (LEE), which plays a key role in the host-cell interactions of EHEC. The activity of LEE1, LEE4, and LEE5 promoters was not significantly altered in *E. coli* O157:H7 strains harboring *toxB* or *efa-1′* mutations, indicating that the effect on the expression of LEE-encoded secreted proteins occurs at a posttranscriptional level. Despite affecting type III secretion, mutation of *toxB* and *efa-1′* did not significantly affect the course of fecal shedding of *E. coli* O157:H7 following experimental inoculation of 10- to 14-day-old calves or 6-week-old sheep. Mutation of *tir* caused a significant reduction in fecal shedding of *E. coli* O157:H7 in calves, indicating that the formation of AE lesions is important for colonization of the bovine intestine.

Enterohemorrhagic *Escherichia coli* (EHEC) strains are zoonotic enteric pathogens of worldwide importance (39). In humans, infection by some EHEC serotypes may cause diarrhea, which may be complicated by hemorrhagic colitis and severe systemic sequelae, including hemolytic-uremic syndrome (45). EHEC strains are closely related to enteropathogenic *E. coli* (EPEC) strains, which are a leading cause of infantile diarrhea in developing countries, and have many of the EPEC genes implicated in virulence (13, 51).

Ruminants are an important reservoir of EHEC (15, 44), and direct or indirect contact with ruminant feces is the leading antecedent to EHEC infection in humans (17, 30, 43). Natural and experimental infections of calves or sheep with EHEC result in efficient colonization of the intestinal tract, and large numbers of bacteria are shed in the feces for several weeks (3, 5–9, 18, 59, 67, 68). Strategies to reduce the prevalence of EHEC in ruminants offer the potential to lower the incidence of human infections (58). However, little is currently known about the mechanisms underlying intestinal colonization of cattle and sheep by EHEC.

EHEC strains are defined by their ability to produce one or more Shiga toxins and to induce characteristic attaching and effacing (AE) lesions on intestinal epithelia, in which the bacteria adhere intimately to the apical surface of enterocytes on raised actin-rich pedestals and microvilli are locally destroyed. This histopathology is determined by the chromosomal locus of enterocyte effacement (LEE), which encodes a type III protein secretion system (13). One of the LEE-encoded secreted proteins (Tir) is translocated into the host cell plasma membrane, where it acts as a receptor for the bacterial outer membrane protein intimin (10). Intimin is an important colonization factor for *E. coli* O157:H7 in neonatal calves (7), lambs (67), and adult cattle and sheep (5). Intimin can also bind to β1-integrins (14) and cell surface-localized nucleolin (57); however, the importance of such interactions and of type III secretion in colonization of the ruminant intestine is unclear. Immunization of calves with *E. coli* O157:H7 type III

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secreted proteins reduces fecal shedding of the bacteria following experimental infection; however, the contribution of individual secreted proteins in colonization and immunity in ruminants requires detailed investigation (50).

Recently, it was reported that the product of the *efa-1* gene (EHEC factor for adherence; referred to in previous publications as *efa1*) influences intestinal colonization in calves by non-O157:H7 EHEC (59). Efa1 was first identified as a factor influencing adhesion of a clinical O111:H$^-$ EHEC strain to cultured epithelial cells (42). EHEC O5 and O111 strains harboring mutations in the *efa-1* gene were shed in the feces in significantly lower numbers than the corresponding parent strains following oral inoculation of conventional calves. Furthermore, an EHEC O111:H$^-$ *efa-1* mutant was impaired in the ability to adhere to the colonic epithelium in calves, as determined by confocal microscopy and direct recovery of bacteria from the mucosa (59).

The mechanism by which Efa1 influences intestinal colonization remains obscure. Mutation of *efa-1* in EHEC O5 and O111 reduces the expression and secretion of LEE-encoded effector proteins, suggesting that it may indirectly influence colonization (59). The EHEC O111:H$^-$ Efa1 protein is 97.4% identical at the amino acid level to EPEC lymphostatin (LifA), which confers an ability to inhibit lymphocyte proliferation of human peripheral blood lymphocytes and the mitogen-activated synthesis of proinflammatory cytokines (25). LifA also inhibits the proliferation of human and murine intraepithelial lymphocytes, indicating that LifA and Efa1 may influence intestinal colonization by modulating mucosal immunity in the gut (21, 25–27, 32).

The *efa-1*/*lifA* gene is present in all non-O157:H7 EHEC serotypes tested so far and in related enteropathogens, such as *Citrobacter rodentium* and rabbit EPEC (REPEC) (25, 42, 60). Sorbitol-fermenting *E. coli* O157:H$^-$ strains also contain *efa-1*; however, the intact *efa-1* gene is absent from the *E. coli* O157:H7 strains that have been sequenced (19, 22, 47). The *efa-1* gene in REPEC was recently shown to mediate bacterial adherence to cultured epithelial cells, apparently without affecting the secretion of LEE-encoded proteins, implying that it may act as an adhesin in its own right (2). While *E. coli* O157:H7 lacks the full-length *efa-1* gene, a truncated version of *efa-1* (*efa-1′*) is present in the chromosome (*z4332* and *z4333* in O island 122 of the EDL933 genome). An *E. coli* O157:H7 mutant carrying a transposon insertion upstream of *efa-1′* (G2-B12) showed reduced adherence to human colon carcinoma cells (62; I. Tatsuno, personal communication), indicating that the truncated Efa1 protein may have some of the properties of full-length Efa1. Recent surveys of the prevalence of O island 122 revealed that it is frequently associated with EHEC isolates from serious and/or epidemic disease, and the authors speculated that the truncated *efa-1* gene may contribute to virulence (24, 38).

In addition to the product of the truncated *efa-1* gene, *E. coli* O157:H7 also encodes a large predicted protein (ToxB/L7095) that exhibits 28% amino acid identity to Efa1 on the pO157 large plasmid (4, 31). *E. coli* O157:H7 strains containing derivatives of pO157 that lack *toxB* exhibit reduced adherence to cultured epithelial cells, possibly as a result of reduced expression and secretion of LEE-encoded type III secreted proteins (63). The *toxB* gene has also been suggested to be a functional homolog of EPEC lymphostatin, since an *E. coli* O157:H7 strain with pO157 deleted lacked the ability to inhibit interleukin-2 and interleukin-4 synthesis in mitogen-activated human peripheral blood lymphocytes (25). However, pO157 encodes other secreted cytotoxins, including enterohemolysin (EhxA), a serine protease (EspP), and a metalloprotease (StcE), and it is unclear if ToxB alone mediates the inhibitory effect.

In order to clarify the role of the *E. coli* O157:H7 *toxB* and truncated *efa-1* genes, we investigated the effect of single and combined defined mutations on the transcription and translation of LEE genes and intestinal colonization in calves and sheep.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. *E. coli* O157:H7 strain 85-170 is a spontaneous *stx1*- and *stx2*-negative derivative of strain 84-289 (65). 85-170 Nal^r is a spontaneous nalidixic acid-resistant derivative of 85-170 that was selected by plating ca. 10^9 CFU of 85-170 on Luria-Bertani (LB) agar containing 25 μg of nalidixic acid per ml, followed by overnight incubation at 37°C. 85-170 Nal^r exhibits normal growth and adhesion characteristics in vitro. Plasmids pCVD442 (11) and pDM4 (37) are positive-selection suicide vectors containing a *pir*-dependent R6K origin of replication, the *mob* region from plasmid RP4, and the *Bacillus subtilis sacBR* genes and confer resistance to ampicillin and chloramphenicol, respectively. pCVD442, pDM4, and derivatives of these plasmids were maintained in *E. coli* DH5α λ*pir* (49) and in *E. coli* S17-1λ*pir* for conjugation (56). Plasmids pAJR71 and pAJR74 have been described previously (52) and contain fusions of the LEE1 and LEE4 promoters, respectively, to a promoterless gene encoding an extended-half-life green fluorescent protein (eGFP). Plasmid pAJR70 (52) has single BamHI and KpnI sites 5′ of a promoterless copy of the gene encoding eGFP and was used to create an LEE5 reporter construct. Bacteria were isolated on LB agar and were cultivated in LB broth with appropriate antibiotics at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 25 μg/ml; and nalidixic acid, 25 μg/ml. For oral inoculation studies bacteria were amplified in brain heart infusion broth for 18 h at 37°C, and the absorbance at 600 nm was adjusted to within 0.05 U.

Cell lines. HeLa cells (ATCC CCL2) were cultivated in RPMI 1640 buffered with 2 g of sodium bicarbonate per liter and supplemented with 10% (vol/vol) fetal calf serum (PAA Laboratories GmbH, Haidmannweg, Austria) and 0.3 g of L-glutamine per liter. For adhesion assays and fluorescent actin staining, cells were seeded at a concentration of 2×10^5 cells/35-mm dish on glass coverslips and grown for 18 h at 37°C in a humidified 5% CO_2 atmosphere.

Routine DNA manipulation. Standard procedures were used for DNA extraction, cloning, PCR, and verification of mutants by Southern hybridization (54).

Construction of defined *E. coli* O157:H7 mutants. A deletion of the entire *toxB* gene was created by overlapping PCR followed by allelic exchange with the positive-selection suicide vector pCVD442. Sequences flanking the *E. coli* O157:H7 *toxB* gene were separately amplified by PCR with Vent proofreading DNA polymerase (New England Biolabs, Beverly, Mass.) by using the primer pairs *tox1* (5′ ATATATGTCGACCGCCACAAAATGGCCG 3′)-*tox2* (5′ GGA AATCAGCGCTACATATCTATACCCTC 3′) and *tox3* (5′ GGGTATAGATA TGTAGCGCTGATTTCCTG 3′)-*tox4* (5′ ATATATGTCGACGAGAGCATG TGCTGGC 3′) (based on the pO157 sequence; accession number AF074613). The primary PCR products were gel purified and combined in an overlapping PCR (20) by using the flanking primers *tox1* and *tox4*. The secondary PCR product was then cloned into pCVD442 via SalI sites incorporated into the primers. The resulting plasmid, pCVDΔ*toxB*, was introduced into 85-170 Nal^r by conjugation from *E. coli* S17-1λ*pir*, and merodiploids were isolated on LB agar containing ampicillin and nalidixic acid. Double recombinants were selected by growing merodiploids to the late logarithmic phase in LB medium lacking ampicillin and plating onto LB agar (without NaCl) containing 6% (wt/vol) sucrose at 30°C. Sucrose-resistant colonies were screened for deletions by colony PCR, and recombinants were verified by Southern hybridization. The deletion resulted in juxtaposition of the predicted start and stop codons of *toxB*.

To create a deletion of the *E. coli* O157:H7 truncated *efa-1* gene, sequences flanking the gene were separately amplified by PCR with Vent DNA polymerase by using the primer pairs *efa-10* (5′ ATATATGAGCTCGATGGTCAGGTC GCAG 3′)–*efa-11* (5′ GGCAGGATAGTTCAATTACATTTCCGCTTAAA 3′) and *efa-12* (5′ GGAAATGTAATTGAACTATCCTGCCGCC 3′)–*efa-13* (5′ AT ATATGAGCTCCTTAGTTCCCTGTAAGCC 3′) (based on the *E. coli*

O157:H7 EDL933 sequence; accession number AE005174). The primary PCR products were gel purified and combined in an overlapping PCR by using the flanking primers *efa-10* and *efa-13*. The secondary PCR product was then cloned into pDM4 via SacI sites incorporated into the primers. The resulting plasmid, pDM4*efa-1′*10+13, was introduced into 85-170 Nal^r and 85-170 Nal^r Δ*toxB* by conjugation from *E. coli* S17-1λ*pir*, and merodiploids were isolated on LB agar containing chloramphenicol and nalidixic acid. Double recombinants were selected essentially as described above, and they were screened for the *efa-1′* deletion by colony PCR and verified by Southern hybridization. The deletion removed the open reading frame encoding the N-terminal 433 amino acids of Efa1′ and also removed the start codon and N-terminal 97 amino acids of a contiguous 3′ open reading frame encoding a predicted 275-amino-acid protein that was 100% identical to amino acids 435 to 710 of Efa1.

A *tir* deletion mutant of strain 85-170 Nal^r was used as a control in calf infection studies and was constructed as follows. Sequences flanking the *tir* gene were separately amplified by using the primer pairs *tir1* (5′ ATATATGAGCTC TAGCATCATCGAGAGGG 3′)-*tir2* (5′ CCTATTGGTAATCTTGGATCCCA TCGTTTCGTC 3′) and *tir3* (5′ GAAACGATGGGATCCAAGATTACCAAT AGGCAT 3′)-*tir4* (5′ ATATATGAGCTCGGGATAACCTTGTCAGG 3′). The primary PCR products were gel purified and combined in an overlapping PCR by using the flanking primers *tir1* and *tir4*. The secondary PCR product was then cloned into pDM4 via SacI sites incorporated into the primers, and the resulting plasmid, pDM4Δ*tir*, was introduced into 85-170 Nal^r by conjugation from *E. coli* S17-1λ*pir*. A double recombinant was selected as described above. The in-frame deletion resulted in juxtaposition of the first and last six codons of the *tir* gene. The 85-170 Nal^r Δ*tir* mutant strain expressed normal levels of intimin, as assessed by Western blotting (data not shown), indicating that the deletion was nonpolar.

Adhesion and fluorescent actin staining assays. Adhesion of EHEC strains to HeLa cells was quantified by visual observation essentially as described previously (16, 63). Semiconfluent HeLa cells grown on 22-mm glass coverslips were overlaid with serum-free RPMI 1640 buffered with 2 g of sodium bicarbonate per liter and were inoculated at a multiplicity of infection of approximately 50:1 with fresh stationary-phase LB medium cultures of 85-170 Nal^r wild-type and mutant strains adjusted to the same optical density. Cells were incubated for 3 h at 37°C in a humidified 5% CO_2 atmosphere, washed three times with 2 ml of phosphate-buffered saline (PBS), and then incubated in fresh medium for an additional 3 h. Cells were washed five times with 2 ml of PBS, fixed, and stained with Hemacolor rapid staining solutions (Merck, Darmstadt, Germany), and multiple images were captured at a magnification of ×400 by using a Leica DMLS microscope with a Polaroid digital microscope camera. For each independent assay 20 randomly selected fields containing 50 or more cells were examined, and the mean number of microcolonies (MC) (comprising eight or more bacteria) per cell was determined. Assays were performed three times, and the microscopic observations were made by a researcher unfamiliar with the identities of the strains tested. The results are given below as the mean number of MC per cell (± standard deviation) from three independent experiments.

Fluorescent actin staining for the detection of filamentous actin under sites of bacterial adhesion to HeLa cells was performed as described previously (28), except that bacteria were incubated on the cells for a total of 8 h.

Detection of EspD by Western blotting. To measure expression and secretion of the LEE-encoded type III secreted protein EspD, bacteria were grown to the late logarithmic phase (A_{600}, ca. 1.0) in minimal essential medium buffered with 25 mM HEPES (MEM-HEPES). Secreted proteins were precipitated with trichloroacetic acid as described previously (35), and total protein was prepared from 1 ml of each culture by centrifugation and resuspension in sodium dodecyl sulfate-polyacrylamide gel electrophoresis sample buffer. Approximately 25 μg of whole-cell protein was analyzed by Western blotting by using a monoclonal antibody specific for EspD and a horseradish peroxidase-conjugated goat anti-mouse immunoglobulin secondary antibody (Dako, Cambridge, United Kingdom). Bound antibody was visualized after 5 min of incubation with a 1:1 mixture of Pierce (Rockford, Ill.) luminol enhancer solution and Pierce stable peroxide solution. Relative levels of EspD were determined by quantifying luminescence with a Flowgen MultiImage cabinet and MultiImage Spot densitometer software (Flowgen, Ashby de la Zouch, United Kingdom). Three independent experiments were performed, and equivalent loading of proteins was verified by Coomassie blue staining of gels.

Detection of EspA filaments by immunofluorescence microscopy. To detect EspA filaments, bacteria grown overnight in M9 minimal medium containing 0.2% (wt/vol) glucose were subcultured in MEM-HEPES. Since EspA filament expression is growth phase dependent (52), cultures were sampled at A_{600} values of 0.4, 0.8, and 1.0. Bacteria were fixed with 0.1% (wt/vol) paraformaldehyde for 5 min at room temperature, and a 20-μl aliquot of each suspension was dried onto a microscope slide at 37°C for 20 min. A 1:100 dilution of EspA antibody (66) in PBS containing 0.1% bovine serum albumin was applied to the slide. After incubation in a humid chamber with gentle rocking for 1 h, the slide was washed three times with PBS containing 0.1% (wt/vol) bovine serum albumin, and then a 1:500 dilution of fluorescein isothiocyanate-conjugated anti-rabbit immunoglobulin secondary antibody was applied to the slide. After incubation and washing as described above, the slides were examined by fluorescence microscopy by using appropriate filter sets, and the images were captured by using Leica software. The number of bacteria stained with the EspA antibody was expressed as a proportion of ca. 900 bacteria from three independent experiments (three fields containing ca. 100 bacteria captured per time point per strain in each of three experiments), essentially as described previously (52).

Measurement of LEE promoter activity by using green fluorescent protein plasmid reporter constructs. Reporter constructs to quantify the activities of the LEE1 and LEE4 promoters (pAJR71 and pAJR74) have been described previously (52). To create an LEE5 reporter construct, the LEE5 (*tir*) promoter was amplified by PCR by using Lee5 5′ BamHI (5′ CGGGATCCCGCGATAAAG AAACTTAATAAACT 3′) and Lee5 3′ KpnI (5′ CGGGTACCTGAAGGTAA TGGAGGTGCAGG 3′) and was cloned into pAJR70 upstream of a promoterless copy of the gene encoding eGFP, yielding pAJR75. To measure the activities of the LEE1, LEE4, and LEE5 promoters, plasmids pAJR71, pAJR74, and pAJR75 were separately transformed into the 85-170 Nal^r wild-type strain and Δ*toxB* and Δ*efa-1′* mutant strains by electroporation by using a Bio-Rad Gene Pulser according to the manufacturer's instructions. Transformants were grown overnight in MEM-HEPES containing chloramphenicol and then subcultured 1:50 in fresh, prewarmed medium. The A_{600} of each of the cultures was monitored by measurement with a spectrophotometer. At intervals, 200-μl portions of the cultures were removed, and the eGFP that had accumulated was quantified by using a FLUOstar Optima fluorescence plate reader (BMG Labtechnologies GmbH, Offenburg, Germany) at appropriate excitation and emission wavelengths. Control bacteria containing no plasmid were used to measure the background fluorescence.

Oral inoculation of calves. Fifteen 10- to-14-day-old Friesian bull calves were used in this study. The calves were housed in a high-containment accommodation in tanks on tenderfoot mats and were fed milk replacer twice daily, and they had free access to water. Prior to infection the calves were confirmed to be culture negative for EHEC and *Salmonella* by direct plating of rectal swabs on sorbitol MacConkey agar containing 2.5 μg of potassium tellurite (T-SMAC) per ml and brilliant green agar (Oxoid, Basingstoke, United Kingdom), respectively. While is it acknowledged that T-SMAC may not detect all EHEC, this medium is highly selective for serogroups O157 and O26, which are by far the most prevalent serogroups in cattle in the United Kingdom at present. Presumptive EHEC colonies were screened for *stx1* and *stx2* genes by PCR by using the primer pairs Stx1F (5′ ATAAATCGCCATTCGTTGACTAC 3′)-Stx1R (5′ AGAACGCCC ACTGAGATCATC 3′) and Stx2F (5′ GGCACTGTCTGAAACTGCTCC 3′)-Stx2R (5′ TCGCCAGTTATCTGACATTCTG 3′). Animals excreting *stx*-positive *E. coli* were excluded from the study. All calves were obtained from the same farm and received colostrum from their dams for the first 24 to 48 h. After this, no further colostrum was given. Total serum immunoglobulin levels were measured when the calves were 1 or 2 days old as a measure of colostrum intake by the zinc sulfate turbidity assay. Only calves with a zinc sulfate turbidity value of more than 10 were used.

Calves were orally challenged with ca. 1×10^{10} CFU of EHEC in 20 ml of antacid (5% [wt/vol] MgO, 5% [wt/vol] Mg trisilicate, and 5% [wt/vol] $NaHCO_3$ in double-distilled water) just before the morning feeding. Mutants 85-170 Nal^r Δ*toxB*, 85-170 Nal^r Δ*efa-1′*, and 85-170 Nal^r Δ*toxB* Δ*efa-1′* were each administered to two calves. The parent strain, 85-170 Nal^r, was administered to a total of five calves. An 85-170 Nal^r Δ*tir* mutant was administered to four calves. During the next 7 days, the calves were observed for clinical signs, and fecal samples were taken twice daily by rectal palpation. The numbers of viable EHEC per gram of feces were determined by plating triplicate 10-fold serial dilutions onto sorbitol MacConkey agar containing 20 μg of nalidixic acid per ml and 2.5 μg of potassium tellurite per ml (lower limit of detection, 100 CFU/g).

Oral inoculation of sheep. Eighteen 6-week-old cross-bred lambs were randomly divided into two equal groups and supplied with food and water ad libitum, and they were confirmed to be free of EHEC by enrichment culturing as described previously (67). Nine lambs were dosed orally by gavage with 1×10^9 CFU of 85-170 Nal^r resuspended in 10 ml of PBS, and a separate group of nine lambs was given a matching dose of 85-170 Nal^r Δ*toxB* Δ*efa-1′*. Approximately 24 h after administration of the bacteria and as required thereafter for up to 24 days, rectal fecal samples were collected from each group and used for direct plating onto sorbitol MacConkey plates supplemented with 20 μg of nalidixic

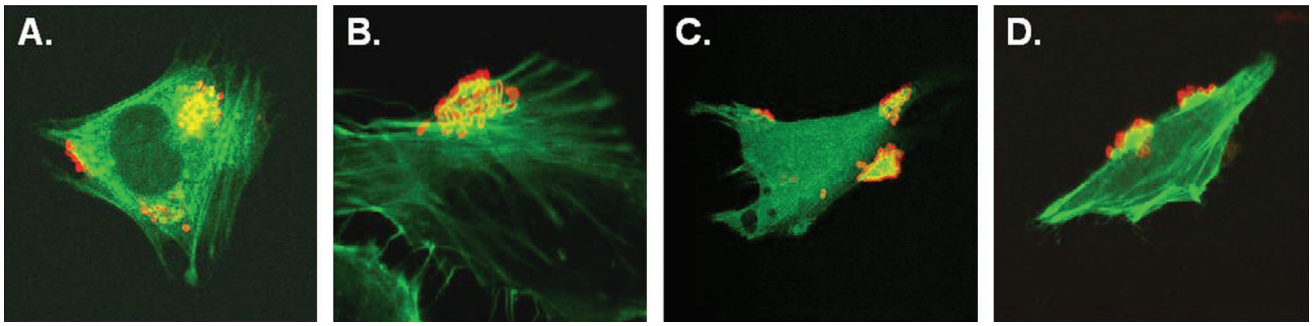

FIG. 1. Nucleation of filamentous actin under sites of adherence of the 85-170 Nal^r wild-type strain and Δ*toxB* and Δ*efa-1* single and double mutant strains to cultured HeLa cells. Cells were examined by fluorescent actin staining by using Oregon Green 514-phalloidin (green) and a confocal laser scanning microscope. Bacteria were detected with rabbit anti-O157 lipopolysaccharide typing serum and anti-rabbit Ig-Alexa^{568} (red). (A) 85-170 Nal^r; (B) 85-170 Nal^r Δ*toxB*; (C) 85-170 Nal^r Δ*efa-1′*; (D) 85-170 Nal^r Δ*toxB* Δ*efa-1′*. Representative images are shown. Magnification, ×630.

acid per ml. One lamb from each group was sacrificed on days 3, 8 and 18 postinfection for bacteriological and histological analyses of tissues.

All animal experiments were performed in accordance with the Animals (Scientific Procedures) Act 1986 and were approved by the local ethical review committee. The fecal shedding data were statistically analyzed for the effect of mutation by using an F test, with the data taken as repeated measurements (Proc Mixed Statistical Analysis System, 1995; SAS Institute, Cary, N.C.). *P* values of <0.05 were considered significant.

RESULTS

Mutation of the *E. coli* O157:H7 *toxB* and truncated *efa-1* genes influences bacterial adherence to cultured HeLa cells but not filamentous actin nucleation. Quantitative adherence assays were performed by using HeLa cells, the 85-170 Nal^r Δ*toxB* and Δ*efa-1′* single and double mutants, and the parent strain. Mutation of the *E. coli* O157:H7 *toxB* and *efa-1′* genes did not alter the in vitro growth rates of the mutants compared to those of the parent strain (data not shown). The numbers of MC comprising eight or more bacteria per HeLa cell in three independent experiments were as follows: for 85-170 Nal^r, 0.0784 ± 0.0162 MC/cell; for 85-170 Nal^r Δ*toxB*, 0.0192 ± 0.0228 MC/cell; for 85-170 Nal^r Δ*efa-1′*, 0.0216 ± 0.0250 MC/cell; and for 85-170 Nal^r Δ*toxB* Δ*efa-1′*, 0.0066 ± 0.0078 MC/cell (means ± standard deviations). Thus, mutation of *toxB* or *efa-1′* alone caused a ca. fourfold reduction in MC formation (for *toxB*, $P = 0.006$; for *efa-1′*, $P = 0.007$). These phenotypes are comparable to those reported by Tatsuno et al. (62, 63). A double mutant lacking both *toxB* and *efa-1′* exhibited a ca. 12-fold reduction in MC formation compared to the wild-type strain ($P = 0.002$). Despite forming fewer MC, the mutant strains still nucleated filamentous actin under the sites of attachment to HeLa cells (Fig. 1), indicating that LEE-mediated rearrangement of cytoskeletal actin could still occur.

We are aware that unintended secondary mutations can result from allelic exchange when positive-selection suicide vectors are used (23), and therefore we attempted to *trans*-complement the defects in MC formation by cloning and expression of the *toxB* and *efa-1′* genes. Several unsuccessful attempts were made to subclone *toxB* on a ca. 10-kb AatII fragment from pO157 into the low-copy-number vector pACYC184. We obtained the mini-pO157 derivative containing *toxB* described by Tatsuno et al. (63) (pIC37, kindly supplied by C. Sasakawa, University of Tokyo). However, this plasmid could not be stably maintained in 85-170 Nal^r Δ*toxB*. When kanamycin-resistant bacteria containing pIC37 were obtained, they exhibited an extremely low growth rate in vitro (data not shown), which prohibited the development of MC in the adherence assay.

Badea et al. recently reported that they were unable to clone the N-terminal 376 amino acids of REPEC *efa-1* (2), presumably because of toxicity. We were successful in cloning the truncated *efa-1* gene from *E. coli* O157:H7 (*z4332*) together with the contiguous open reading frame *z4333* in pET30-Ek/Lic (Novagen, Madison, Wis.). However, induction of expression of *efa-1′* caused a dramatic decrease in the growth rate, making quantification of MC formation unreliable (data not shown).

Mutation of the *E. coli* O157:H7 *toxB* and truncated *efa-1* genes reduces expression and secretion of LEE4-encoded proteins. Expression and secretion of the LEE4-encoded type III secreted protein EspD by the *E. coli* O157:H7 wild-type strain and Δ*toxB*, Δ*efa-1′*, and Δ*toxB* Δ*efa-1′* mutant strains were analyzed by Western blotting by using specific antiserum (Fig. 2). The densities of secreted EspD based on three independent experiments were as follows: for 85-170 Nal^r, 1,664 ± 108; for 85-170 Nal^r Δ*toxB*, 792 ± 123; for 85-170 Nal^r Δ*efa-1′*, 1,638 ± 196; and for 85-170 Nal^r Δ*toxB* Δ*efa-1′*, 201 ± 87 (means ± standard deviations). Thus, mutation of *toxB* caused a signifi-

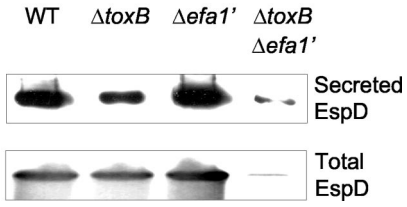

FIG. 2. Effects of *toxB* and *efa-1′* mutations on expression and secretion of the LEE4-encoded type III secreted protein EspD. Whole-cell and secreted protein fractions of 85-170 Nal^r, 85-170 Nal^r Δ*toxB*, 85-170 Nal^r Δ*efa-1′*, and 85-170 Nal^r Δ*toxB* Δ*efa-1′* (ca. 25 μg of protein) were resolved by sodium dodecyl sulfate–15% polyacrylamide gel electrophoresis. Proteins were transferred to nitrocellulose membranes and probed by using a mouse monoclonal antibody specific for EspD. Equivalent loading of proteins was confirmed by Coomassie blue staining of gels (data not shown). WT, wild type.

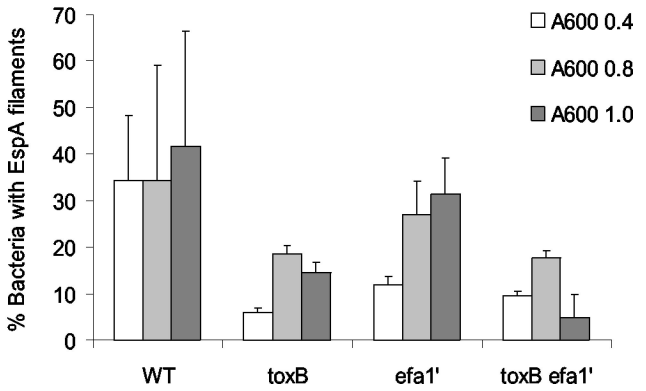

FIG. 3. Effect of mutation of *toxB* and *efa-1′* on the formation of EspA filaments. EspA filaments were visualized by indirect immunofluorescence following subculture of bacteria grown in M9 minimal medium into MEM-HEPES. Samples were prepared at A_{600} values of 0.4, 0.8, and 1.0. The proportions of bacteria expressing EspA filaments were determined as described in Materials and Methods, and the data are the means ± standard deviations for three independent experiments. WT, wild type.

cant reduction in the secretion of EspD (the value was 2.1-fold lower than the wild-type value; $P = 0.001$). While mutation of *efa-1′* alone had little effect ($P = 0.82$), a cumulative reduction in EspD expression occurred when both *toxB* and *efa-1′* were deleted (the value was ca. 8.2-fold lower than the wild-type and single *efa-1′* mutant values; $P < 0.001$). Similar reductions in the expression of EspD were detected following analysis of whole-cell protein from the wild-type and mutant strains (Fig. 2).

We also analyzed the expression of EspA filaments by the wild type and the Δ*toxB* Δ*efa-1′* mutant strain by indirect immunofluorescence. It was observed previously that EspA filaments are not produced during growth in M9 minimal medium but are induced following subculture in MEM-HEPES (52). Upon induction, not all bacteria in the population express EspA filaments at a given time, and the highest proportion of bacteria with EspA filaments is detected in the late exponential phase (52). We determined the proportion of wild-type and mutant bacteria elaborating EspA filaments at A_{600} values of 0.4, 0.8, and 1.0. Mutation of *toxB* and *efa-1′* singly or in combination resulted in a reduction in the proportion of bacteria elaborating EspA filaments (Fig. 3); however, the difference was significant only for samples collected at an A_{600} of 0.4 (for 85-170 Nal^r Δ*toxB*, $P = 0.004$; for 85-170 Nal^r Δ*efa-1′*, $P = 0.013$; for 85-170 Nal^r Δ*toxB* Δ*efa-1′*, $P = 0.008$). During the late logarithmic phase (A_{600}, 0.8 and 1.0), the extent of EspA filamentation observed with the mutant strains was consistent with the levels of EspD detected by Western blotting (Fig. 2). Since the production of EspA filaments by EHEC is dependent on EspD (29), it is not possible to state whether the observed reduction in EspA filament production in the mutant strains was the result of lower EspD production (Fig. 2), lower EspA production, or a combination of the two.

Since production of LEE4-encoded proteins is growth phase dependent (52, 53), it was not possible to *trans*-complement the defects in EspD or EspA production owing to the inhibitory effect of plasmids containing *toxB* or *efa-1′* on bacterial growth (data not shown).

Mutation of the *E. coli* O157:H7 *toxB* and truncated *efa-1* genes does not affect the activity of the LEE1, LEE4, and LEE5 promoters. To determine if mutation of *toxB* and *efa-1′* impairs the production of EspD and EspA filaments by reducing the transcription of LEE genes, we separately transformed a series of plasmid-borne eGFP-reporter constructs into the wild-type and mutant strains in order to measure the activity of the LEE1 (*ler*), LEE4 (*sepL*), and LEE5 (*tir*) promoters (pAJR71, pAJR74, and pAJR75, respectively). The emission of fluorescence due to accumulation of eGFP was quantified at several points in the growth cycle. No significant differences in the activities of the LEE1, LEE4, and LEE5 promoters were detected in strains harboring the *toxB* and *efa-1′* mutations compared to the parent strain (Fig. 4). Indeed, the activity of the LEE4 promoter was slightly greater for the 85-170 Nal^r Δ*toxB* and Δ*efa-1′* mutant strains than for the parent strain. Thus, mutation of *toxB* and *efa-1′* affects the expression of EspD at a posttranscriptional level.

Mutation of the *E. coli* O157:H7 *toxB* and truncated *efa-1* genes does not affect intestinal colonization in calves or sheep. To investigate the role of the *E. coli* O157:H7 *toxB* and *efa-1′* genes in intestinal colonization, we first inoculated two 10- to 14-day-old conventional Friesian bull calves with *E. coli* 85-170 Nal^r Δ*toxB*, 85-170 Nal^r Δ*efa-1′*, or 85-170 Nal^r Δ*toxB* Δ*efa-1′* by the oral route. Colony PCRs were performed on days 1 and 7 postinoculation by using a minimum of 10 independent nalidixic-acid resistant isolates from each calf, and this analysis confirmed that only the inoculum strain was shed (data not shown). The *stx*-negative 85-170 Nal^r parent strain was administered to five calves and was shed at levels of $>10^5$ CFU/g of feces for the duration of the experiment. No significant differences in the shedding patterns were detected between the wild-type and mutant strains (*P* values at 7 days postinoculation: for Δ*toxB*, 0.83; for Δ*efa-1′*, 0.10; and for Δ*toxB* Δ*efa-1′*, 0.9) (Fig. 5). As a control, four calves were inoculated with an 85-170 Nal^r mutant harboring an in-frame nonpolar deletion in the *tir* gene. By day 7 postinoculation the 85-170 Nal^r Δ*tir* mutant was shed at significantly lower numbers than the wild type ($P = 0.004$). Indeed, the reduction in shedding of the *tir* mutant reached significance at just 3 days postinoculation ($P = 0.018$).

We separately assessed the ability of the 85-170 Nal^r Δ*toxB* Δ*efa-1′* mutant to colonize the ovine intestine by orally inoculating groups of nine 6-week-old sheep with the wild-type or double mutant strain, and fecal shedding was quantified for up to 24 days. This model was recently used to establish a role for intimin in persistent colonization of the ovine intestine (67). No significant differences in the shedding patterns were detected between the wild-type and mutant strains at any time tested (Fig. 6).

DISCUSSION

It has been reported previously that mutation of the *efa-1* gene of EHEC serotypes O5 and O111 results in a significant reduction in fecal shedding of the organisms ($P < 0.05$ at 7 days postinoculation) (52). *E. coli* O157:H7 strains lack the full-length *efa-1* gene; however, a truncated version of *efa-1* is

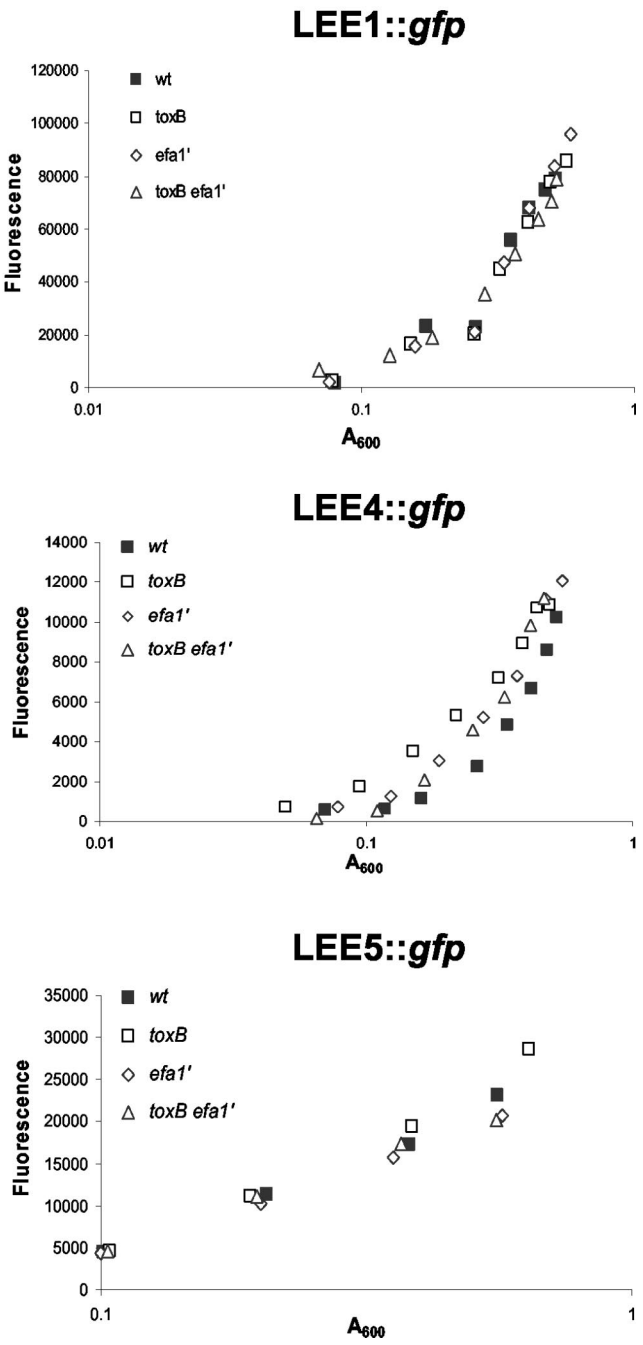

FIG. 4. Effect of *toxB* and *efa-1′* mutations on the activity of LEE1, LEE4, and LEE5 promoters. Plasmids pAJR71 (*lee1*::*gfp*), pAJR74 (*lee4*::*gfp*), and pAJR75 (*lee5*::*gfp*) were separately transformed into 85-170 Nal^r, 85-170 Nal^r Δ*toxB*, 85-170 Nal^r Δ*efa-1′*, and 85-170 Nal^r Δ*toxB* Δ*efa-1′*, and the bacteria were grown in MEM-HEPES. At stages in the growth cycle the absorbance of the cultures was recorded, and the fluorescence due to accumulation of eGFP was quantified. The data are the means for two independent measurements. *wt*, wild type.

present in the chromosome (19, 47), and a homolog of *efa-1* (*toxB*/*l7095*) is present in the large plasmid (4, 31). To clarify the role of these genes, we constructed single and double Δ*toxB* and Δ*efa-1′* mutants of *E. coli* O157:H7 and assessed

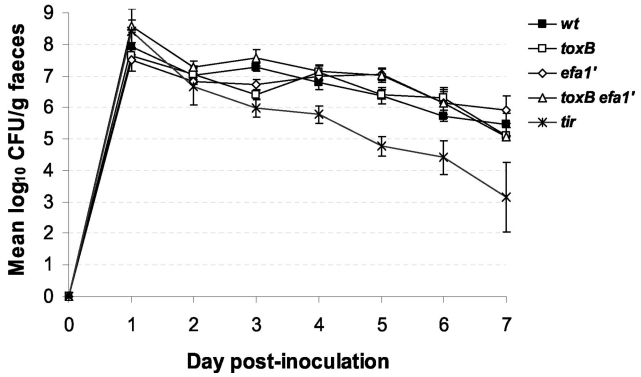

FIG. 5. Mutation of *toxB* and *efa-1′* does not significantly affect the course of fecal shedding of *E. coli* O157:H7 in calves. The data are fecal shedding data for duplicate calves inoculated with the 85-170 Nal^r Δ*toxB*, 85-170 Nal^r Δ*efa-1′*, and 85-170 Nal^r Δ*toxB* Δ*efa-1′* mutants. Data from five calves infected with 85-170 Nal^r and four calves infected with 85-170 Nal^r Δ*tir* are shown for comparison. The bacterial recovery from the feces was measured twice each day. The data are daily means ± standard errors of the means. *wt*, wild type.

the phenotypes of the mutants in vitro and in calves and sheep.

Consistent with the observations of Tatsuno et al. (63), we found that deletion of the *E. coli* O157:H7 *toxB* gene led to a reduction in the expression and secretion of LEE4-encoded type III secreted proteins and impaired MC formation on cultured epithelial cells. Furthermore, we found for the first time that a defined mutation of the truncated version of *efa-1* alone caused a reduction in MC formation on HeLa cells. This is consistent with the finding that a transposon insertion upstream of *efa-1′* reduced adherence to Caco-2 cells to 25 to 50% of the wild-type adherence level (62; I. Tatsuno, personal communication). The *efa-1′* single mutant expressed and secreted wild-type levels of EspD, and the production of EspA filaments was not reduced significantly during the phase of growth when such filaments are expressed maximally. This is in accordance with the finding that the G2-B12 *efa-1′* mutant

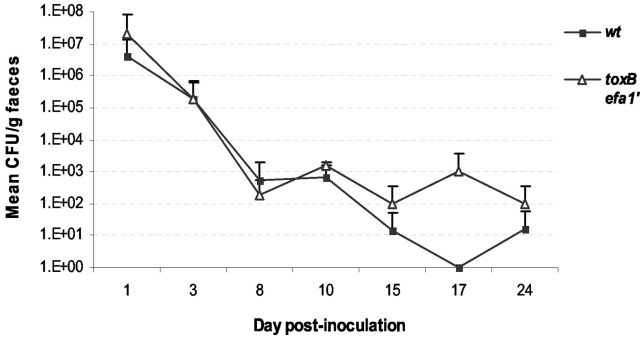

FIG. 6. Mutation of both *toxB* and *efa-1′* does not significantly affect the course of fecal shedding of *E. coli* O157:H7 in 6-week-old sheep. Animals were inoculated with 85-170 Nal^r or 85-170 Nal^r Δ*toxB* Δ*efa-1′* (nine animals per group), and fecal samples were collected at intervals up to 24 days. One lamb from each group was sacrificed on days 3, 8, and 18 postinfection, and postmortem examinations were performed. The data are means ± standard deviations for different times.

identified by Tatsuno et al. produced wild-type levels of EspA as assessed by immunoblotting (62). In contrast, deletion of *efa-1′* in combination with *toxB* led to marked reductions in the expression and secretion of EspD and EspA filament production and a further reduction in the ability of the bacteria to form MC on cultured cells, implying that there is a synergistic effect. In all cases the reduction in EspD and EspA expression and secretion was not sufficient to prevent the nucleation of filamentous actin under the sites of *E. coli* O157:H7 attachment to cultured cells (62, 63; this study). Some bacteria harboring the Δ*toxB* Δ*efa-1′* double mutation still assembled EspA filaments on their surfaces, as detected by indirect immunofluorescence. Since actin condensation under adherent bacteria is dependent on the delivery of LEE-encoded type III secreted effectors, our data indicate that sufficient quantities of LEE4-encoded proteins are still produced and translocated into epithelial cells so that the bacteria can elicit cytoskeletal rearrangements.

We attempted to *trans*-complement the defects in MC formation and the expression and secretion of EspD and EspA in the mutant strains by cloning and expressing the *toxB* and *efa-1′* genes. Several unsuccessful attempts were made to subclone *toxB* from pO157, and it is likely that this gene is unstable and/or toxic, as has been reported for the homologous 9.5-kb *efa-1* gene (22, 25, 42). A mini-pO157 derivative containing *toxB* described by Tatsuno et al. (63) could not be stably maintained in 85-170 Nal^r Δ*toxB*, most likely as a result of incompatibility with the endogenous pO157Δ*toxB* plasmid. We were able to clone the truncated *efa-1* gene from *E. coli* O157:H7; however, induction of gene expression restricted the growth rate, probably as a consequence of toxicity, as reported elsewhere (2).

Mutation of *toxB* caused a defect in the expression and secretion of LEE-encoded type III secreted effectors whose magnitude was similar to that seen in EHEC O5 and O111 *efa-1* mutants (59). Type III secretion of EspD was impaired by ca. eightfold in an *E. coli* O157:H7 mutant harboring deletions of both *toxB* and *efa-1′*. Despite this, no significant reduction in fecal shedding of *E. coli* O157:H7 was seen in calves or sheep infected with the mutant strains. Our data are consistent with the observation that an *E. coli* O157:H7 strain with the pO157 plasmid which encodes *toxB* deleted colonizes gnotobiotic piglets and induces enteritis at least as well as the wild type (65). We cannot preclude the possibility that there were differences in the immune status of the animals used in the present study or the possibility that various levels of passively acquired EHEC-specific maternal antibody masked subtle effects of the mutations.

LEE-encoded type III secreted proteins are major virulence factors for several AE pathogens in their target animal species, including EPEC EspB in humans (61), REPEC EspA, EspB, and Tir in rabbits (1, 33), and *Citrobacter rodentium* EspB in mice (41). Data supporting a role for LEE-encoded type III secreted proteins in the colonization of ruminants by *E. coli* O157:H7 were recently provided by the finding that immunization of calves with *E. coli* O157:H7 type III secreted proteins reduces fecal shedding of the bacteria upon subsequent challenge (50). Considerable natural variation in the level of secretion of the LEE-encoded Tir and EspD proteins has been observed among EHEC O157:H7 strains isolated from cattle (35); however, it is not known if this correlates with the ability of the strains to colonize the intestines efficiently. While the *E. coli* O157:H7 Δ*toxB* Δ*efa-1′* double mutant was impaired in the ability to express and secrete LEE4-encoded proteins, the amount of EspD secreted by this mutant was comparable to the amounts secreted by naturally occurring *E. coli* O157:H7 strains isolated from cattle (35; Roe and Gally, unpublished observations).

When the ability of *E. coli* O157:H7 to form AE lesions was abolished through deletion of the *tir* gene, we observed a significant reduction in fecal shedding of the bacteria following oral inoculation of calves. This indicates that differences in the ability of EHEC strains to colonize the bovine intestine can be detected with our oral challenge model and that the model does not merely measure shedding of the inoculum. Furthermore, it shows for the first time that the translocated intimin receptor is required for intestinal colonization in calves. This implies that the role of intimin in intestinal colonization of calves and sheep may be explained, at least in part, by its ability to bind to its translocated receptor instead of secondary receptors, such as β1-integrins (14) and/or cell surface-localized nucleolin (57). There is strong evidence for the existence of host cell intimin receptors, and the role of such molecules in the carriage and tissue tropism of EHEC merits close investigation.

It is unclear why mutation of *efa-1* in EHEC O5 and O111 impairs intestinal colonization in calves whereas mutation of *toxB* in EHEC O157:H7 does not, even though type III secretion is similarly affected in the two backgrounds. It is possible that different EHEC serotypes rely to different extents on the LEE-encoded type III secretion system for intestinal colonization. There is increasing evidence that different EHEC serovars colonize the intestines of calves by distinct mechanisms. We and other workers have shown that EHEC serotypes O5, O26, and O111 adhere extensively to the intestinal epithelium in the bovine cecum, colon, and rectum and can readily be seen forming AE lesions (46, 59). In contrast, *E. coli* O157:H7 is rarely detected in association with the intestinal epithelium at these sites following oral inoculation of calves even though high numbers of organisms may be shed (6, 8, 68). *E. coli* O157:H7 may exhibit a specific tropism for lymphoid follicle-dense mucosa at the terminal rectum in weaned calves and adult cattle (40); however, preliminary data from our laboratories have indicated that non-O157 EHEC serotypes do not share a tropism for this site (S. W. Naylor, A. J. Roe, and D. L. Gally, unpublished observations). It is not known if *E. coli* O157:H7 exhibits a specific tropism for the terminal rectum in animals that are the ages we used. Indeed, the preweaned calves and 6-week-old lambs inoculated in this study had not started to ruminate, and it is not clear if lymphoid follicle-dense epithelium has even matured in the terminal rectum at this age. The role of specific *E. coli* O157:H7 genes in colonization of the terminal rectum and other sites in preweaned, weaned, and adult ruminants awaits detailed investigation.

It is possible that Efa1 influences intestinal colonization of calves by EHEC O5 and O111 by acting independently of the LEE. Given that Efa1 is 97.4% identical to EPEC lymphostatin, this molecule may facilitate colonization by suppressing the activation of gastrointestinal lymphocytes. However, in a recent study it was not possible to detect lymphostatin-like effects

on bovine intraepithelial lymphocytes exposed to EHEC serotype O103:H2 in situ in a ligated ileal loop model of infection (36). It is also possible that Efa1 acts as an adhesin per se or influences adhesion indirectly by some other mechanism. Indeed, Badea et al. reported that Efa1 mediates adherence of REPEC to cultured epithelial cells, apparently without affecting the expression and secretion of LEE-encoded proteins (2). Furthermore, Efa1-specific antibody inhibited the adherence of REPEC to cultured cells (2). It may be that ToxB and the truncated version of Efa1 do not influence intestinal colonization by *E. coli* O157:H7 because they do not share the activities of Efa1.

Our data and those of Tatsuno et al. (63) suggest that Efa1 and ToxB influence the expression of LEE-encoded type III secreted proteins at a posttranscriptional level. Almost all LEE-positive strains tested to date contain either Efa1 or ToxB (25, 42, 60), and indeed in EHEC O26 strain 413/89-1 and REPEC strains RDEC-1 and 83/89, the LEE and *efa-1* are adjacent on the chromosome (64, 69) (EHEC O26 LEE accession number AJ277443). This close genetic linkage occurs in many other EPEC and EHEC strains (38) and may imply that Efa1 is required for complete activity of the LEE-encoded type III secretion system. Furthermore, O island 122, which contains truncated *efa-1′*, is also significantly associated with LEE-positive EHEC strains that cause serious and/or epidemic disease (24, 38). No enzymatic activities have yet been ascribed to the EHEC Efa1, Efa1′, and ToxB proteins. Efa1 and ToxB are highly homologous to large clostridial toxins at the N terminus and share a DXD glycosyltransferase motif (4, 25, 31, 42), and both contain a cysteine protease motif that is present in numerous other secreted bacterial virulence factors (55). The 433-amino-acid truncated Efa1 protein lacks both these motifs, and their relevance to the activities of Efa1 and ToxB remains to be tested.

The mechanism by which Efa1 and ToxB influence the translation of LEE4 transcripts is unclear. For some type III secretion systems in gram-negative bacteria, it has been suggested that the secretion signal for translocated effector proteins is present in the mRNA and that translation and secretion of the proteins may be coupled at the needle complex (48). If such a situation applied to the EHEC LEE-encoded type III secretion apparatus, then it is conceivable that *toxB* and *efa-1′* mutations may influence expression and secretion of LEE4-encoded proteins by interfering with the proper insertion and assembly of the type III secretion apparatus in the membrane and/or the targeting of LEE transcripts.

Interestingly, LifA does not appear to be required for efficient production and secretion of LEE-encoded effector proteins in human or REPEC strains (2, 59). It is noteworthy that the cloned EPEC LEE can confer AE activity upon *E. coli* K-12 (34), whereas the cloned LEE from *E. coli* O157:H7 cannot (12), suggesting that there are differences in the control of type III secretion between the strains. The involvement of Efa1, Efa1′, and ToxB in this process is the subject of ongoing studies in our laboratory.

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